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Published in:
Advanced Synthesis & Catalysis

DOI:
[10.1002/adsc.200900176](https://doi.org/10.1002/adsc.200900176)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Hellemond, E. W., Vermote, L., Koolen, W., Sonke, T., Zandvoort, E., Heuts, D. P. H. M., Janssen, D. B., & Fraaije, M. W. (2009). Exploring the Biocatalytic Scope of Alditol Oxidase from *Streptomyces coelicolor*. *Advanced Synthesis & Catalysis*, 351(10), 1523-1530. <https://doi.org/10.1002/adsc.200900176>

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Exploring the Biocatalytic Scope of Alditol Oxidase from *Streptomyces coelicolor*

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Received: March 19, 2009; Revised: May 19, 2009; Published online: June 30, 2009

Abstract: The substrate scope of the flavoprotein alditol oxidase (AldO) from *Streptomyces coelicolor* A3(2), recombinantly produced in *Escherichia coli*, was explored. While it has been established that AldO efficiently oxidizes alditols to D-aldoses, this study revealed that the enzyme is also active with a broad range of aliphatic and aromatic alcohols. Alcohols containing hydroxy groups at the C-1 and C-2 positions like 1,2,4-butanetriol ($K_m=170$ mM, $k_{cat}=4.4$ s⁻¹), 1,2-pentanediol ($K_m=52$ mM, $k_{cat}=0.85$ s⁻¹) and 1,2-hexanediol ($K_m=97$ mM, $k_{cat}=2.0$ s⁻¹) were readily accepted by AldO. Furthermore, the enzyme was highly enantioselective for the oxidation of 1,2-diols [e.g., for 1-phenyl-1,2-ethanediol the (*R*)-enantiomer was preferred with an *E*-value of 74]. For sev-

eral diols the oxidation products were determined by GC-MS and NMR. Interestingly, for all tested 1,2-diols the products were found to be the α -hydroxy acids instead of the expected α -hydroxy aldehydes. Incubation of (*R*)-1-phenyl-1,2-ethanediol with ¹⁸O-labelled water (H₂¹⁸O) revealed that a second enzymatic oxidation step occurs *via* the hydrate product intermediate. The relaxed substrate specificity, excellent enantioselectivity, and independence of coenzymes make AldO an attractive enzyme for the preparation of optically pure 1,2-diols and α -hydroxy acids.

Keywords: alcohol oxidation; alditol oxidase; biocatalysis; 1,2-diols; α -hydroxy acids

Introduction

The oxidation of primary and secondary alcohols to their corresponding carbonyl compounds is one of the most important reactions in synthetic organic chemistry, since both the alcohol and its oxidation product(s) are widely used as building blocks for further synthetic purposes. Chemical methods for alcohol oxidation are based on heavy metals like chromium or manganese, which are often used in stoichiometric amounts. Recently, catalytic aerobic oxidation methods have been developed, which are more efficient and less polluting but require multiple (inorganic) components and often lack selectivity.^[1]

Biocatalytic oxidations have the advantages of being environmentally friendly, catalytically efficient and often exhibit exquisite regio- and enantioselectiv-

ity.^[2] Several oxidizing enzymes can be applied for the selective oxidation of alcohols. Alcohol dehydrogenases (ADHs), catalyzing the oxidation of an alcohol into the corresponding aldehyde or ketone using NAD(P)⁺ as electron acceptor, are used extensively.^[3] They are abundant; several ADHs acting on different primary and secondary alcohols have been described and are commercially available.^[4] Moreover, ADHs are often very regio- and enantioselective, making them suitable for (dynamic) kinetic resolution processes. A major drawback of ADHs is their dependence of expensive coenzymes like NAD(P)⁺.

In contrast to ADHs, alcohol oxidases (AOs) use the cheap and clean oxidant molecular oxygen as an electron acceptor, which is reduced to H₂O₂. The latter can easily be decomposed to O₂ and H₂O by employing a catalytic amount of catalase. AOs are

much less abundant compared to ADHs. Most of them are of eukaryotic origin, which can make efficient overexpression of the AO encoding genes a tedious task. AOs can also act on a wide range of compounds like carbohydrates,^[5] aliphatic alcohols,^[6] aromatic alcohols^[7] and steroids.^[8] However, their use for synthetic purposes is still limited when compared to the extensive number of oxidase-based biosensing and diagnostic applications.

In a search for novel AOs we recently identified an oxidase primarily acting on alditols.^[9] This enzyme was isolated from the actinomycete *Streptomyces coelicolor* A3(2) and was named alditol oxidase (AldO). AldO is a 45-kDa soluble monomeric flavoprotein and contains covalently bound FAD as a cofactor. In order to produce AldO in significant amounts, the AldO encoding gene was fused to that of maltose binding protein (MBP), which resulted in expression levels of AldO in *Escherichia coli* of up to 350 mg of protein per liter of culture. Initial substrate profiling of AldO showed that the oxidase was primarily active with alditols like xylitol ($K_m=0.32$ mM, $k_{cat}=13$ s⁻¹) and sorbitol ($K_m=1.4$ mM, $k_{cat}=17$ s⁻¹) and that the primary alcohol group was oxidized, resulting in, respectively, the aldoses D-xylene and D-glucose as products. Recently, the three-dimensional structure of AldO in complex with several alditols was solved.^[10]

To further investigate the biocatalytic potential of AldO for the selective oxidation of alcohols, we studied the substrate scope and selectivity of AldO more extensively. In this paper we show that the substrate specificity of AldO is not restricted to alditols as the oxidase is active with a broad range of alcohols. Also we show that AldO is highly regio- and enantioselective for the oxidation of 1,2-diols. Surprisingly, not the α -hydroxy aldehydes but the α -hydroxy carboxylic acids were formed as the products in the latter reaction.

Results

Effect of pH and Temperature on AldO Activity and Stability

To establish the best conditions for performing biocatalysis with AldO, some of its stability characteristics were determined. This revealed that AldO exhibits a broad pH optimum displaying a relative activity of >80% between pH 6 to 9. AldO shows a temperature optimum for enzyme activity of 55 °C. The enzyme is relatively stable with a half-life of 40 h at 37 °C and 4 h at 50 °C. Under conditions which were applied for the oxidation experiments [10 μ M of AldO in 50 mM sodium phosphate buffer, pH 7.5, at 30 °C and rotary shaking (100 rpm)] AldO has a half-life of 30 h.

Substrate Specificity of AldO

Previously it was found that AldO was primarily active with alditols, with xylitol being the best substrate.^[9] To investigate whether the substrate specificity of AldO was restricted to alditols, a broad range of compounds was tested using a qualitative 96-well plate assay. At a substrate concentration of 10 mM and using 12 nM of AldO, the enzyme only showed activity with alditols, while at higher substrate and enzyme concentrations (50 mM and 1.2 μ M, respectively), activity was detected for aldoses, 1,2-diols, aliphatic primary and secondary alcohols and *cis*-2-butene-1,4-diol.

For all identified substrates the steady state kinetic parameters were determined (Table 1). Xylitol is the best substrate with a k_{cat} of 13.0 s⁻¹ (17.3 U/mg) and a K_m of 0.32 mM. Besides alditols, 1,2-diols are reasonable substrates indicating that two adjacent hydroxy groups at C-1 and C-2 seem to be a minimal requirement for a compound in order to be effectively oxi-

Table 1. Steady-state kinetic parameters of AldO at pH 7.5.

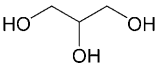
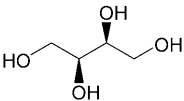
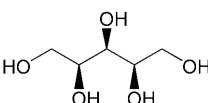
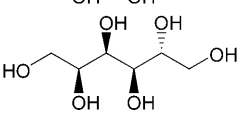
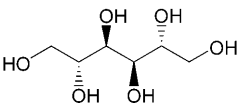
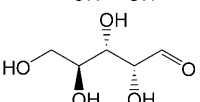
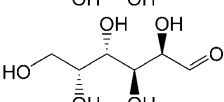
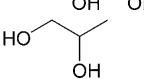
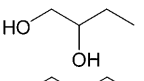
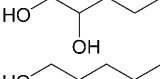
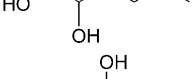
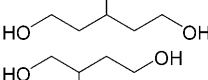
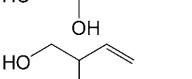
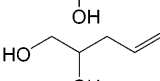
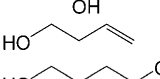
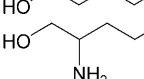
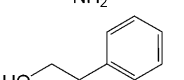
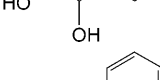
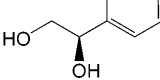
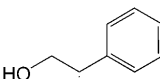
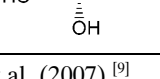
| Substrate | Structural formula | K_m [mM] | k_{cat} [s ⁻¹] | k_{cat}/K_m [s ⁻¹ M ⁻¹] |
|---------------------------|---|-----------------|------------------------------|--|
| glycerol |  | 350 \pm 50 | 1.6 \pm 0.1 | 4.6 |
| L-threitol ^[a] |  | 25 \pm 1 | 6.3 \pm 0.1 | 250 |
| xylitol ^[a] |  | 0.32 \pm 0.03 | 13.0 \pm 0.2 | 41,000 |
| D-sorbitol ^[a] |  | 1.4 \pm 0.1 | 17.0 \pm 0.2 | 12,000 |

Table 1. (Continued)

| Substrate | Structural formula | K_m [mM] | k_{cat} [s ⁻¹] | k_{cat}/K_m [s ⁻¹ M ⁻¹] |
|-----------------------------------|---|------------|------------------------------|--|
| D-mannitol ^[a] |  | 36 ± 2 | 9.2 ± 0.2 | 260 |
| L-arabinose |  | 430 ± 40 | 1.7 ± 0.1 | 4.0 |
| D-galactose ^[b] |  | — | — | 0.3 |
| 1,2-propanediol ^[b] |  | — | — | 0.3 |
| 1,2-butanediol |  | 150 ± 20 | 0.29 ± 0.01 | 1.9 |
| 1,2-pentanediol |  | 52 ± 4 | 0.85 ± 0.03 | 16 |
| 1,2-hexanediol |  | 97 ± 11 | 2.0 ± 0.1 | 21 |
| 1,3,5-pentanetriol ^[b] |  | — | — | 7.8 |
| 1,2,4-butanetriol |  | 170 ± 10 | 4.4 ± 0.1 | 26 |
| 3-butene-1,2-diol |  | 250 ± 40 | 0.34 ± 0.02 | 1.4 |
| 4-pentene-1,2-diol |  | 42 ± 5 | 0.35 ± 0.01 | 8.3 |
| 3-butenol |  | 480 ± 70 | 0.10 ± 0.1 | 0.2 |
| 1,4-butanediol ^[b] |  | — | — | 0.5 |
| 2-amino-1-pentanol ^[c] |  | 35 ± 8 | 0.017 ± 0.001 | 0.6 |
| 1-phenyl-1,2-ethanediol |  | 83 ± 9 | 0.36 ± 0.01 | 4.3 |
| (R)-1-phenyl-1,2-ethanediol |  | 101 ± 1 | 0.740 ± 0.004 | 7.3 |
| (S)-1-phenyl-1,2-ethanediol |  | 86 ± 6 | 0.0080 ± 0.0002 | 0.1 |

^[a] Values were taken from Heuts et al. (2007).^[9]

^[b] Due to a high K_m , only the k_{cat}/K_m value could be determined.

^[c] The pH of the substrate stock solution was adjusted to 7.5 by adding HCl.

dized by AldO. For example, 1,2,4-butanetriol is readily accepted by AldO, while 1,4-butanediol is a very poor substrate. However, aldoses (also matching the requirement of adjacent hydroxy groups at C-1 and C-2, like D-galactose and L-arabinose) are poor substrates. This can be explained by the fact that in an

aqueous solution only a small percentage of the aldoses will be in the linear free aldehyde form (which can be accepted by AldO) while the majority will be in the pyranose or furanose form, which excludes oxidation of the terminal hydroxy group.

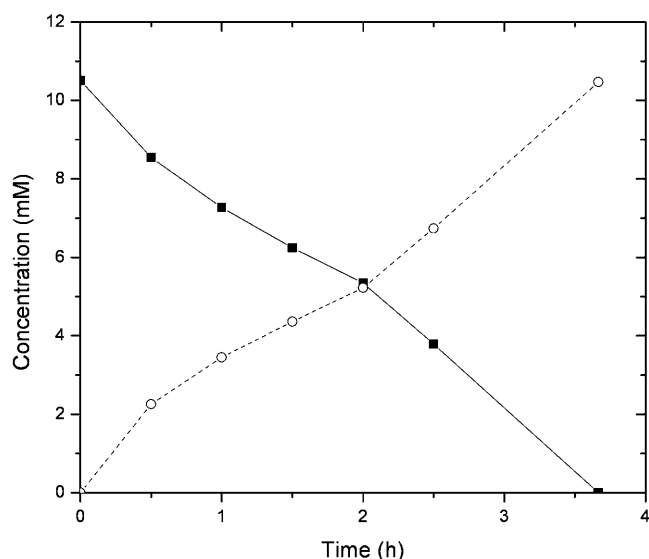


Figure 1. AldO-catalyzed oxidation of xylitol (■) to D-xylose (○). Conditions: 10 mM xylitol, pH 7.5, room temperature, 3.24 U/mL catalase, 0.24 μ M AldO.

AldO-Catalyzed Oxidation of Polyols

The AldO-catalyzed oxidation of alditols revealed that the enzyme is strictly acting on the primary hydroxy group of the substrate. Oxidation of this group results in the formation of the corresponding D-aldoses.^[9] To investigate the applicability of AldO as an oxidative biocatalyst for small-scale conversions we first followed the conversion of xylitol over time. This revealed a complete conversion to D-xylose after 4 h, while no by-products were formed (Figure 1). A slight decline in conversion rate after the start of the batch reaction may suggest some oxygen diffusion. The steady formation of D-xylose is in line with the fact that aldoses do not inhibit AldO.

To obtain more insight into the biocatalytic potential of the enzyme we investigated the enantioselectivity and product formation for a series of chiral substrates. For this we incubated two aliphatic diols (1,3-butanediol, 1,2-pentanediol) and an aromatic diol (1-phenyl-1,2-ethanediol) with AldO and analyzed the formed products by TLC, GC-MS and NMR (Table 2). By GC-MS analysis we could identify 3-hydroxybutanal as the product of 1,3-butanediol. The products of 1,2-pentanediol and 1-phenyl-1,2-ethanediol were identified by NMR. Surprisingly not the aldehydes but the α -hydroxy acids were found to be the products of these substrates. For 1,2-pentanediol 50% of the diol was converted after 22 h into α -hydroxypentanoic acid and for 1-phenyl-1,2-ethanediol 35% conversion to mandelic acid and two minor by-products (<5%) was observed after 65 h. The formation of the α -hydroxy acids rather than the expected α -hydroxy aldehydes as oxidation products corresponds

Table 2. Product identification of AldO catalyzed oxidations.

| Substrate | Product |
|-------------------------|---------|
| 1,3-butanediol | |
| 1,2-pentanediol | |
| 1-phenyl-1,2-ethanediol | |

[a] Identified by GC-MS.

[b] Identified by NMR.

well with the high polarity of the products observed by TLC.

Chiral GC analyses of AldO-mediated conversions of 1,2-pentanediol and 1-phenyl-1,2-ethanediol revealed a clear change in the enantiomeric excess of the remaining substrate (ee_s), reaching ee_s values of >95% and 43%, respectively (50 and 34% conversion, respectively). This suggests that only one enantiomer of these substrates remained in the reaction mixture, while the other was effectively converted. Clearly, besides being stereoselective towards alditols,^[9,10] AldO is an enantioselective biocatalyst for the kinetic resolution of racemic 1,2-diols. For the conversion of 1-phenyl-1,2-ethanediol by AldO an E -value of 74 can be calculated from the ratio between the specificity constants (k_{cat}/K_m) of the separate enantiomers (Table 1). These kinetic data also reveal the preference of AldO for the (*R*)-enantiomer of this aromatic diol. From the degree of conversion of 1,2-pentanediol and ee_s ^[11] an E -value of >145 can be calculated for this aliphatic diol.

Mechanism of α -Hydroxy Acid formation by AldO

The formation of α -hydroxy acids instead of the α -hydroxy aldehydes as the AldO oxidation products of 1,2-diols triggered us to investigate the mechanism behind this phenomenon. Since isolated enzyme was used, an aldehyde oxidase side activity of *E. coli* could be ruled out. The most reasonable options for the α -hydroxy acid formation are 1) the spontaneous chemical oxidation of the initially formed aldehyde to the acid by oxygen from air, or 2) a second enzymatic oxidation of the aldehyde *via* the hydrate (*gem*-diol) (Figure 2).

To distinguish between these two mechanisms we analyzed the conversion of (*R*)-1-phenyl-1,2-ethanediol to (*R*)-mandelic acid in the presence of ^{18}O -labelled water (H_2^{18}O). In the first-mentioned mecha-

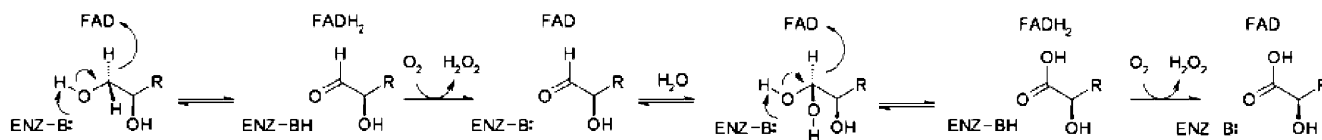


Figure 2. Proposed mechanism for the oxidation of 1,2-diols to the α -hydroxy acids ($R = C_3H_7$, C_4H_9 , Ph). In the first step the diol is oxidized by AldO to the α -hydroxy aldehyde, while FAD is reduced to $FADH_2$. In water, the α -hydroxy aldehyde is in equilibrium with its hydrate (*gem*-diol), which can be oxidized in a second step to the α -hydroxy acid.

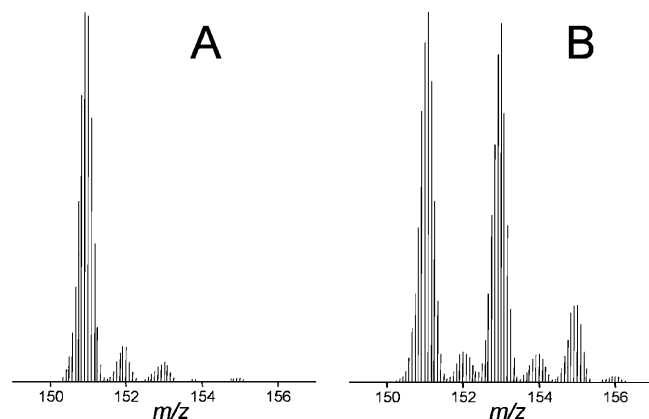


Figure 3. (A) ESI-MS (negative ion mode) of the oxidation products of the AldO-catalyzed oxidation of (*R*)-1-phenyl-1,2-ethanediol. (B) Spectrum in presence of $H_2^{18}O$. The peak at $m/z = 151$ corresponds to deprotonated (*R*)-mandelic acid. The peaks at $m/z = 153$ and $m/z = 155$ correspond to the deprotonated forms of (*R*)-mandelic acid with the incorporation of one and two ^{18}O atoms, respectively.

nism, this would not result in the presence of ^{18}O in the product. The second mechanism would result in $H_2^{18}O$ hydrating the aldehyde yielding the ^{18}O -containing hydrate (*gem*-diol), which can subsequently be oxidized a second time by AldO, resulting in the presence of ^{18}O in the product.

MS analysis of the reaction products of the AldO-catalyzed oxidation of (*R*)-1-phenyl-1,2-ethanediol in the presence of 33% (v/v) of $H_2^{18}O$ revealed, besides the expected peak at $m/z = 151$ [corresponding to the deprotonated form of (*R*)-mandelic acid], a clear peak at $m/z = 153$ and a small peak at $m/z = 155$, corresponding to the deprotonated forms of (*R*)-mandelic acid containing, respectively, one and two ^{18}O atoms per molecule (Figure 3). In the control samples there was only a major peak at $m/z = 151$, as expected. The incorporation of ^{18}O atoms in the product supports a mechanism in which a second enzymatic oxidation step occurs *via* the initially formed hydrate product (Figure 2).

Conclusions

In this paper we discuss the biocatalytic scope of alditol oxidase from *Streptomyces coelicolor* (AldO). AldO displays a relaxed substrate specificity since, besides alditols, the enzyme accepts aliphatic and aromatic 1,2-diols as a substrate, albeit with a poor catalytic efficiency. Both for alditols and aliphatic 1,2-diols the k_{cat} increases with the carbon chain length, while the lowest K_m is observed for a chain length of 5 C-atoms. However, the K_m values for the 1,2-diols are considerably affected by the absence of additional hydroxy groups. This suggests that formation of multiple hydrogen bonds is important for productive substrate binding^[10].

This study reveals that AldO is an enantioselective oxidative biocatalyst. For 1-phenyl-1,2-ethanediol clearly the (*R*)-enantiomer is preferred. Likewise, the (*R*)-enantiomer of aliphatic 1,2-diols is expected to be the fast reacting enantiomer. Inspection of the crystal structure of AldO complexed with alditols indicates that these polyols are bound *via* an extensive hydrogen bonding network^[10] (Figure 4, *left*). The C-2-OH group in xylitol makes hydrogen bonding interactions with Ser106 and Glu320. Binding of (*R*)-1-phenyl-1,2-ethanediol will enable formation of similar interactions (Figure 4, *right*). Contrarily, binding of (*S*)-1-phenyl-1,2-ethanediol would preclude formation of these hydrogen bonds while His343 will sterically hinder positioning of the C-2-OH. As a result the substrate will bind in an improper way to facilitate oxidation: transfer of a hydride from C-1 to the N-5 of the FAD cofactor.

Product analysis of AldO-catalyzed oxidation reactions revealed that the α -hydroxy acids rather than the α -hydroxy aldehydes were formed. $H_2^{18}O$ labeling experiments revealed that the observed acid formation is enzyme-catalyzed (Figure 2). For AldO, two cycles of oxidation do not occur with alditols as substrate, since ring closure of the initially formed aldose will prevent hydrate formation. Also 1,3-butanediol is merely oxidized into the corresponding aldehyde. This indicates that the double oxidation of, for example, 1-phenyl-1,2-ethanediol into the corresponding acid can be considered as catalytically specific towards 1,2-diol substrates. Thus, this enzyme is able to perform oxidations of alcohols into aldehydes/ketones

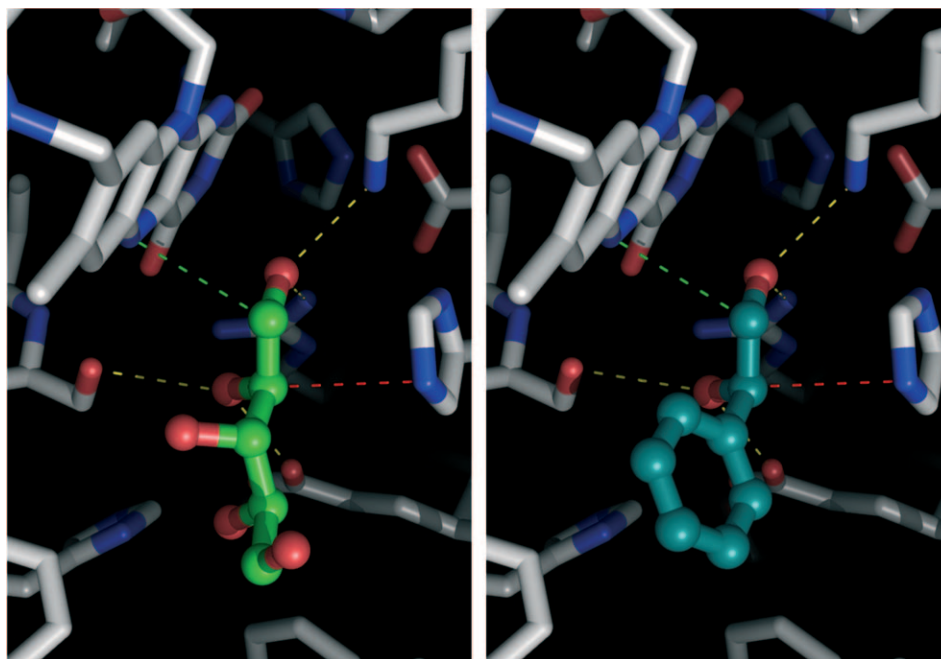


Figure 4. Active site of AldO with xylitol as observed in the crystal structure (*left*), and with manually docked (*R*)-1-phenyl-1,2-ethanediol (*right*). Hydrogen bonds involving C-1 and C-2 hydroxy groups are indicated in yellow, the N-5–C-1 distance in green and the His343–C-2 distance in red.

(single oxidation) and oxidations of alcohols into acids (double oxidation), depending on the specific substrate. The double oxidation of a primary alcohol by a flavin-containing oxidase yielding the carboxylic acid as a product has also been reported for a glycopeptide oxidase.^[12]

The preparation of optically pure diols is of great importance for the synthesis of chiral drugs.^[13] So far kinetic resolution approaches for the oxidation of 1,2-diols include the oxidation of (*S*)-1,2-phenylethanol by *Enterobacter aerogenes* glycerol dehydrogenase^[14] and the oxidation of aliphatic (*S*)-1,2-diols by *Rhodobacter sphaeroides* galactitol dehydrogenase^[15] and *Hansenula ofunaensis* glycerol dehydrogenase.^[16] In contrast to these enzymes, AldO from *S. coelicolor* oxidizes (*R*)-1,2-diols to α -hydroxy acids, with no formation of the ketone, while the reaction does not require expensive coenzymes. These catalytic properties render AldO an efficient oxidative biocatalyst in kinetic resolution of racemic diols.

Experimental Section

Materials

Glycerol, xylitol, (\pm)-1-phenyl-1,2-ethanediol, (\pm)-butene-1,2-diol, (\pm)-1,2,4-butanetriol, L-(+)-leucinol and (\pm)-2-amino-1-pentanol were purchased from Acros. (\pm)-1,2-Hexanediol, (*R*)-1-phenyl-1,2-ethanediol and (*S*)-1-phenyl-1,2-ethanediol were obtained from Aldrich. L-Threitol, 1,4-butanediol, (\pm)-1,2-pentanediol, 3-buten-1-ol and 2,2'-azino-

bis(3-ethylbenzothiazoline-6-sulfonic acid) diazonium salt (ABTS), horseradish peroxidase (HRP; EC 1.11.1.7) and catalase from bovine liver (EC 1.11.1.6) were from Fluka. D-Galactose, D-sorbitol and (*R*)-mandelic acid were obtained from Janssen Chimica. D-Mannitol was from J.T. Baker Chemicals BV. L-Arabinose was purchased from Lancaster. 1,3,5-Pentanetriol and (\pm)-4-pentene-1,2-diol were a kind gift from Prof. F. Rutjes (Radboud University Nijmegen, The Netherlands). All other chemicals were of analytical grade.

Expression and Purification of AldO

Alditol oxidase from *S. coelicolor* A3(2) was expressed as an N-terminal MBP fusion protein [alditol oxidase linked at its N-terminus to maltose binding protein (MBP)] in *E. coli* TOP10. The enzyme was purified as described before.^[9] One enzyme U is defined as the amount of enzyme that catalyzes the conversion of 1 μ mol of substrate per minute.

Screening of AldO Substrates

To screen for potential AldO substrates a qualitative 96-well plate assay was used. In total 86 compounds were screened: D-ribose, D-xylose, D-glucose, D-lyxose, lactose, maltose, D-mannose, D-fructose, L-fucose, melizitose, D-galactose, L-arabinose, L-rhamnose, sucrose, D-arabitol, xylitol, D-mannitol, *meso*-erythritol, D-sorbitol, ribitol, galactitol, L-gulonolactone, diglycerol, hexaethylene glycol, diethyleneglycol, ethylene glycol, glycerol, PEG, *N*-acetylglucosamine, sarcosine, L-arginine, L-alanine, cholesterol, D-alanine, L-asparagine, L-aspartate, DL-aspartate, L-cysteine, L-histidine, L-proline, L-threonine, 3-buten-1-ol, 3-buten-2-ol, cis-2-buten-1,4-diol, DL-homoserine, L-threitol, L-ornithine, DL-norvaline,

1,3-propanediol, 2-aminoethanol, 1-amino-2-propanol, 2-amino-1-propanol, 4-amino-1-butanol, 1,2-butanediol, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 1,4-diaminobutane, 2-butanol, 1-pentanol, 2-pentanol, 1-octanol, benzyl alcohol, 2-phenylethanol, isoamylalcohol, α -methylbenzyl alcohol, α -methylbenzyl amine, 1-phenyl-1,2-ethanediol, 1,2-propanediol, 2-amino-1-phenylethanol, D-2-amino-2-phenylethanol, L-2-amino-2-phenylethanol, 1,3-butanediol, benzyl methylether, 1,4-butanediol, cinnamyl alcohol, D-meso-2,3-butanediol, 2-methoxy-4-methylphenol, vanillyl alcohol, 1,2-butanediol, 1,2-pentanediol, 1,2-hexanediol, *cis*-1,2-cyclohexanediol. For substrate profiling, every well (100 μ L) contained 50 mM potassium phosphate (KPi) buffer pH 7.5, 100 μ M ABTS and 50 U mL⁻¹ HRP. The concentrations of AldO and the putative substrates were varied resulting in four different screening conditions: a) 10 mM of putative substrate, 12 nM AldO, b) 50 mM of putative substrate, 12 nM of AldO, c) 10 mM of putative substrate and 1.2 μ M of AldO and d) 50 mM of putative substrate and 1.2 μ M of AldO. For the putative substrate cholesterol the concentrations were 2 μ M cholesterol, also containing 0.2% DMSO for conditions a) and c) and 10 μ M, also containing 1% DMSO for the conditions b) and d). The 96-well plates were incubated at room temperature for 24 h and regularly checked by visual inspection for green colour formation as a result of H₂O₂/HRP-mediated ABTS oxidation.

Enzyme Activity and Determination of Kinetic Parameters

Unless stated otherwise enzyme activity was measured at 25°C in 50 mM KPi buffer pH 7.5 using the H₂O₂ coupled assay as described before^[9] containing 0.1 mM 4-aminoantipyrine (AAP), 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) and 4 U of HRP. Steady-state kinetic parameters were determined by measuring initial rates with different substrate concentrations. Data were fitted with Origin 7.0 using the Michaelis–Menten equation. Three different buffers were used to determine the effect of pH on the enzyme activity: 50 mM citrate buffer (pH 2.5 to 6), 50 mM sodium phosphate buffer (pH 6 to 8) and 50 mM glycine/NaOH buffer (pH 8.6 to 10.6). Initial activity was measured at 25°C using the assay described above with 0.1 mM ABTS instead of AAP/DCHBS as the HRP substrate. In this case 0.1 mM ABTS, 5 U of HRP and 0.012 μ M of AldO were used and the initial activity was calculated from the increase in A₄₂₀ (ϵ_{420} = 36 mm⁻¹ cm⁻¹). At nine different temperatures between 10 and 80°C the initial activity was measured using a temperature-controlled spectrophotometer. The effect of temperature on enzyme stability was determined by incubation of 250 μ L samples of AldO (107 μ M) at 37 and 50°C. Samples were taken and the residual activity was determined with regular time intervals using the AAP/DCHBS activity assay.

AldO-Catalyzed Oxidation Reactions

The oxidation of xylitol was performed in a 100-mL Erlenmeyer flask with 20 mL of 50 mM KPi buffer, pH 7.5, containing 10 mM xylitol, 0.24 μ M AldO and 324 U catalase at room temperature. Samples were taken regularly and analyzed by HPLC for substrate depletion and product formation. Oxidation reactions with racemic 1,3-butanediol

(100 mM), 1,2-pentanediol (50 mM), and 1-phenyl-1,2-ethanediol (50 mM) were performed in a closed 500-mL Erlenmeyer flask containing 10 mL of 50 mM KPi buffer, pH 7.5, 10 μ M AldO and 6800 U catalase. Reactions were performed at 30°C in a shaker-incubator at 100 rpm. Control reactions without AldO were performed in parallel. Samples were taken regularly for the determination of the oxidation product(s) and analysis of the enantiomeric excess of the remaining substrate (*ee*_s).

Oxidation of (*R*)-1-Phenyl-1,2-ethanediol in Presence of H₂¹⁸O

To elucidate the mechanism of the AldO-catalyzed oxidation of 1,2-diols to the corresponding α -hydroxy acids, the oxidation of (*R*)-1-phenyl-1,2-ethanediol was performed with and without H₂¹⁸O. Oxidation experiments were done in 2-mL Eppendorf tubes, in a total volume of 300 μ L 50 mM KPi buffer, pH 7.5, containing 33% (v/v) H₂¹⁸O. The reaction mixture also contained 50 mM (*R*)-1-phenyl-1,2-ethanediol, 7 U mL⁻¹ catalase and 20 μ M AldO. Two control reactions with H₂¹⁸O were performed: one without enzyme and one without substrate, containing 50 mM (*R*)-mandelic acid. The reaction mixtures were incubated overnight at 30°C and then analyzed by MS. MS analysis of oxidation products of 1,2-phenylethanediol was performed on a Thermo LCQ Fleet mass spectrometer. Measurements were performed by direct spray, operating in the negative ion mode using 0.1% formic acid in H₂O:methanol (70:30).

Product Identification

Product identification for the AldO-catalyzed conversion of xylitol was performed by HPLC using a Shodex SUGAR SP0810 column (8.0 mm ID \times 300 mm) at 80°C using H₂O as an eluent with a flow rate of 0.7 mL min⁻¹. For detection an RI detector was used. For product identification of the AldO-catalyzed conversion of racemic diols samples were analyzed by GC-MS (EI). All analyses were performed on a CP-SIL 8CB column (30 m \times 0.25 mm, d_f 0.5 μ m) with the following temperature program: from 40°C to 300°C at 5°C min⁻¹. Helium was used as the carrier gas with a constant flow of 1 mL min⁻¹ and split ratio of 25:1. The injector temperature was 250°C. The mass spectrum was scanned from *m/z* = 15 to 550 with an acquisition rate of 1.8 scans s⁻¹. Products formed upon oxidation of 1,2-pentanediol and 1-phenyl-1,2-ethanediol were analyzed by NMR. 1 mL of sample was lyophilized and redissolved into 0.5 mL of D₂O. Structures were elucidated by means of ¹H, COSY, TOCSY, HSQC and HMBC spectra on a Bruker Avance spectrometer at 600 MHz using standard Bruker pulse programs. The NMR data were also used to monitor the degree of conversion.

Chiral GC Analysis

To determine the enantioselectivity of AldO for chiral diols, samples were analyzed by chiral GC. All samples were diluted 1:1 with acetonitrile before GC analysis, except for 1,3-butanediol, which was further diluted as follows: 100 μ L sample + 400 μ L pyridine + 400 μ L [*N,O*-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS)]. The analyses were performed on a chiral Betadex

column. For 1,3-butanediol and 1,2-pentanediol the following temperature program was used: 1 min at 50 °C, from 50 °C to 180 °C at 5 °C min⁻¹, 5 min at 180 °C. For 1-phenyl-1,2-ethanediol the following temperature program was used: 150 °C to 220 °C at 20 °C min⁻¹, 10 min at 220 °C.

Acknowledgements

This research was financially supported by the Integrated Biosynthesis Organic Synthesis (IBOS) program of the Netherlands Organisation for Scientific Research (NWO). We thank Lucien Duchateau (DSM Pharmaceutical Products) and Dick Schipper (DSM Biotechnology Center, Analysis) for analytical support.

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